Evaluation of Retinal Toxicity and Liposome Encapsulation of the Anti-CMV Drug 2′-nor-cyclic GMP

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Purpose. Human cytomegalovirus (HCMV) is an important pathogen in the immunocompromised patient. CMV retinitis is a leading cause of blindness in patients with AIDS. Ganciclovir and foscarnet are currently the treatments being used for this retinitis, but they both have major toxicities when used systemically. Intravitreal therapy with ganciclovir has been used in some patients who cannot tolerate systemic treatment. The major problem with this modality is the necessity for administration of between 1 and 3 intravitreal injections per eye per week. 2′-nor-cyclic GMP is a nucleotide analog, a cyclic phosphate derivative of ganciclovir. Neutral salts of the compound are extremely water soluble, and the charged phosphate group at neutral pH make it an ideal candidate for encapsulation into a multivesicular liposome system.

Methods. The authors evaluated the retinal toxicity of the diethanolammonium salt 2′-nor-cyclic GMP by using electroretinographic, morphologic, and ophthalmoscopic techniques after intravitreal injections in rabbit eye.

Results. The intraocular therapeutic index for 2′-nor-cyclic GMP is 20. At the 10 μg dose, electroretinogram, ophthalmoscopic examination, and both light and electron microscopy revealed no abnormalities. Toxicity was evident at 50 μg and higher doses with ERG changes (loss of amplitude) and retinal pathology that varied from vacuolization of the retinal pigment epithelium and loss of height of the outer photoreceptor segment to loss of the entire outer retina. In addition, an in vitro drug release half-life of 1,000 hours (more than 75 times that of ganciclovir) was found for 2′-nor-cyclic GMP in liposome, which may be able to be exploited in the therapy of patients with CMV retinitis unable to tolerate toxic systemic therapy.

Conclusion. The anti-CMV drug, 2′-nor-cyclic GMP, may be promising for intravitreal injection, particularly if encapsulated into liposomes. Invest Ophthalmol Vis Sci 1993;34:2903–2910.

Human cytomegalovirus (HCMV) is an important pathogen in the immunocompromised patient, especially in patients with acquired immunodeficiency syndrome (AIDS). Retinitis caused by HCMV infection in patients with AIDS is a leading cause of blindness in this population. Ganciclovir (DHPG), a hydroxylated homolog of acyclovir, is currently the treatment being used for this retinitis. Licensed for use in the United States since 1989, ganciclovir is usually successful in controlling CMV infection, but it is toxic to the bone marrow and some other organ systems so therapy often must be interrupted. Foscarnet has recently been licensed in the United States and has approximately equal efficacy as ganciclovir when used systemically, but it also entails major systemic toxicity, particularly electrolyte imbalance and nephrotoxicity. For these reasons, local intravitreal therapy has been used in patients who cannot tolerate systemic therapy. Intravitreal therapy with ganciclovir is used in some patients as 100 to 200 μg injections on a once-a-week to three-times-a-week basis. The major problem with this modality is the necessity for frequent administration,
which often must be bilateral; thus, patients with bilateral CMV retinitis may receive two to six injections weekly.4,5,6,7

For this reason, we have been interested in anti-CMV compounds that are polar and that may be incorporated into the aqueous phase of liposome systems. This would have the advantage of prolonging the intravitreal half-life of the compound and might allow for a more clinically achievable administration schedule. 2'-nor-cyclic GMP is a nucleotide analog that is the sodium salt of the cyclic phosphate derivative of ganciclovir.8 The compound is extremely water soluble (100 mg/ml); its charged phosphate group would make it an ideal candidate for encapsulation into a multivesicular liposome system, which we have used with other compounds.9,10 The antiviral action of this compound is independent of thymidine kinase12 and appears to have a direct activity on viral DNA polymerase. Most of the antiviral agents (e.g., acyclovir) that have been used for herpes virus infections depend on their phosphorylation by the virus-encoded thymidine kinase for activity. Human CMV, which does not encode for a specific thymidine kinase, does not show any sensitivity to these antivirherpes drugs. 2'-nor-cyclic GMP is active in vitro against thymidine kinase-deficient herpes simplex virus and varicella and those strains that are resistant to the “classical” anti-herpes drugs. Of note, ganciclovir-resistant CMV strains have been isolated from immunocompromised patients treated with ganciclovir for CMV infection.13 As suggested by Biron et al,14 this resistance can be based on the impaired phosphorylation of the drug. It is likely that such strains will be sensitive to the phosphorylated analogs of ganciclovir such as 2'-nor-cyclic GMP, as kinase-dependent herpes resistors were shown to be.14

In animal models of herpes keratitis15 2'-nor-cyclic GMP, appears to be more effective than trifluridine (F3T). It has potent in vitro activity against many DNA viruses, including herpes simplex, HCMV, vaccinia, SV-40, and adenovirus.8 In guinea pig CMV models, it has a selectivity index (50% cytotoxicity dosage in stationary monolayer guinea pig embryo cells/50% effective dose by plaque reduction assay) of 110, which is 10-fold higher than ganciclovir. In plaque reduction assays, 2'-nor-cyclic GMP has approximately 20-fold greater potency against guinea pig CMV than ganciclovir.16 In addition, it appears to have a more prolonged mode of action than does ganciclovir in guinea pig CMV tissue culture systems.16 In HCMV (AD169) models, it is of similar potency to ganciclovir with ID50 (drug concentration inhibiting viral plaque by 50%) of 2 μmol/l compared to 0.43 to 7 μmol/l for ganciclovir.6,17 Compared to foscarcin, 2'-nor-cyclic GMP has 66 times more potency against HCMV and 10 times more potency against HSV-1.8,18

In summary, 2'-nor-cyclic GMP has several properties that make it well suited for intracocular delivery. First, the compound produces a long-lasting antiviral effect that may require a less frequent dosing interval.16 Because of its polar nature, the compound may be encapsulated into liposomes.12 A repository preparation such as liposomes may be advantageous for local intraocular therapy for CMV retinitis. We therefore wished to determine the intraocular toxicity and therapeutic index of 2'-nor-cyclic GMP and its suitability for encapsulation into a multivesicular liposome system. Finally, ganciclovir-resistant CMV strains have been isolated from immunocompromised patients treated with ganciclovir for CMV infection13 and may be sensitive to 2'-nor-cyclic GMP. For these reasons we chose to evaluate the retinal toxicity of 2'-nor-cyclic GMP in the rabbit eye after intravitreal injection.

MATERIALS AND METHODS

Animal Studies

Twenty-five New Zealand white rabbits, each weighing 2.5 to 3 kg, were used. All experimentation was performed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology.

Electroretinography (ERG) was performed on all rabbits before the procedure and before euthanasia at either 30 or 60 days as previously described.19 Before the procedure, the eyes were dilated with 2 to 3 drops of 2.5% phenylephrine and 1% mydriacyl and dark adapted for 30 minutes. A mixture of ketamine/xylazine was used for anesthesia (21 mg/kg ketamine, 5 mg/kg xylazine). The eyes were positioned 6 inches from the Grass stimulator light source. Silver-impregnated nylon electrodes were placed on the corneas. Four to eight flash ERGs were averaged, transferred to an amplifier and DA converter by our design, and then transmitted to a IBM-based personal computer. Data were collected on eyes containing 2'-nor-cyclic GMP or normal saline. Other control data included a data bank of normal ERG amplitudes and latencies in New Zealand White rabbits corrected for age and weight of the animals.

For intraocular injections, the rabbits were anesthetized with intramuscular injections of 28 to 56 mg/kg ketamine and 12 mg/kg xylazine. One percent proparacaine was used for topical anesthesia of the cornea. Under sterile conditions, 0.1 ml of aqueous from the anterior chamber was removed with a 27-gauge needle, which caused a decrease of the intraocular pressure before drug delivery. The rabbits received intravitreal injections of 0.1 ml of 2'-nor-cyclic GMP at doses of either 10, 20, 50, and 100 μg of drug dissolved in normal saline, or normal saline. The drug was injected with a 25-gauge needle 1 to 2 mm posterior to...
the limbus into the vitreous. The tip of the needle was in the mid-vitreous cavity during the injection.

Each rabbit was examined by indirect ophthalmoscopy before injection, at 5 days after injection when applicable, and before euthanasia. At the conclusion of 1, 3, 7, 30, and 60 days, the rabbits were killed and perfusion fixed. All rabbits were deeply anesthetized with 80 mg/kg ketamine and 80 mg/kg xylazine mixture. After intracardiac injection of 1 ml of heparin, 1,000 U, an intra-aortic cannula was inserted and the descending aorta was clamped. Then 1 liter of room temperature normal saline was perfused, followed by 1 liter of fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M Sorenson’s phosphate, pH 7.2. At this time, an incision was made in the right atrium. After the eyes were encuclalated, a small incision was placed at the limbus and the eyes were placed for postfixation in the same fixative in a refrigerator for 1 hour. The samples were then transferred to buffer 0.1 M Sorenson’s phosphate, washed three times, and refrigerated for gross sectioning. The details of the eye numbers and time points are listed in Table 1.

Histopathologic Methods

Gross sectioning of each eye delivered three sections for histology. After bisecting each eye through the optic nerve, one area above and one below the medullary ray were taken from one-half of the globe for electron microscopy. A section was removed from the other half of the globe for thin light microscopy (2 μm sections). All tissues were postfixed in osmium after preliminary embedding of each sample in 2.25% agar. For thin light microscopy, the sections were plastic embedded in glycol methacrylate, and the 2-μm thick sections were stained with toluidine blue. For electron microscopy, the sections were plastic embedded in epon-araldite. Preliminary embedding of each sample in 2.25% agar. For thin light microscopy, the sections were removed from the other half of the globe for thin light microscopy (2 μm sections). All tissues were postfixed in osmium after preliminary embedding of each sample in 2.25% agar. For thin light microscopy, the sections were plastic embedded in glycol methacrylate, and the 2-μm thick sections were stained with toluidine blue. For electron microscopy, the sections were embedded in epon-araldite.

Preparation of Liposomes and Drug Release Studies

Liposomes were prepared as previously described. In brief, 7.5 mg of dioleyl phosphatidyl choline, 1.5 mg of dimyristoyl glycerol, 1.68 mg of triolein, and 5.81 mg of cholesterol were dissolved in 1 ml of chloroform. One milligram per ml solution of 2’-nor-cyclic GMP diethanolammonium salt was dissolved in 200 μmol/l of sucrose at pH 6.8. One milliliter of this solution was added drop-wise to a vial containing the 1 ml of chloroform lipid mixture. The vial was tightly sealed and vortex mixed for 6 minutes. Floating in a pool of chloroform and excess lipids, spherules of water droplets coated with a lipid monolayer were produced. Next, 1 ml of this mixture was added rapidly to each of the two vials containing 2.5 ml of 240 μmol/l of glucose. The new suspension was vortexed for 4 seconds.

Studies

The resulting liposomes were then dried in a flask containing 250 μmol/l of sucrose by passing nitrogen over them at 8 l/min until no detectable chloroform remained. The nitrogen was then passed for an additional 2 minutes. The mixture was centrifuged at 400 × G for 10 minutes, producing a liposomal pellet. To remove unencapsulated drug, the supernatant was withdrawn and the liposomes were resuspended in 0.9% sodium chloride solution and recentrifuged. This procedure was repeated five times to remove any unencapsulated drug. Drug capture efficacy was determined by measuring the percentage of drug amount within liposomes versus that within liposomes and supernatant.

The drug release studies for ganciclovir and 2’-nor-cyclic GMP were performed in triplicate at 37°C with the liposomes suspended in phosphate buffered saline within a 30-ml syringe mounted on a revolving rotor. At designated time intervals, 3 ml aliquots were drawn off and centrifuged. The supernatant was then

<table>
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<th>Drug Concentration (μg/0.1 ml)</th>
<th>Day 1 n/Hist/ERG</th>
<th>Day 3 n/Hist/ERG</th>
<th>Day 7 n/Hist/ERG</th>
<th>Day 30 n/Hist/ERG</th>
<th>Day 60 n/Hist/ERG</th>
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<tr>
<td>Control</td>
<td>1/0/ND</td>
<td>1/0/ND</td>
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<tr>
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<td>2/0/ND</td>
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</tr>
<tr>
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<td>1/1/ND</td>
<td>—/—/—*</td>
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<tr>
<td>100</td>
<td>—/—/—*</td>
<td>2/1/ND</td>
<td>4/3/ND</td>
<td>2/3/3</td>
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n = number of eyes tested.
Hist, histology. 0, no toxicity. 1, mild toxicity consisting of retinal pigment epithelium hyperplasia. 2, moderate toxicity consisting of vacuolization of the retinal pigment epithelium and loss of height of the photoreceptor outer segment. 3, severe toxicity consisting of distortion of the entire outer retina with loss of retinal pigment epithelium and photoreceptor layer and reduction in the outer nuclear layer. ERGs, electroretinogram, dark adapted. 0, normal waveform, amplitude, and latency. 3, severe loss of amplitude and abnormal waveforms. ND, not done. ERGs were not performed within 2 wk of injection because of nonspecific changes seen in our laboratory after intraocular injections of any substance. 9

* No data.
FIGURE 1. Intravitreal injection of 10 μg 2-nor-cyclic GMP in rabbit eye 2 months earlier. (A) Electroretinogram before and after (arrow) injection shows normal waveform, amplitude, and latency. (B) Light micrograph of methacrylate-embedded retina, the cytoarchitecture of the entire retina, appears normal. G = ganglion cell layer, P = photoreceptor layer, arrows = RPE. (Counterstained with toluidine blue X600). (C) Electron micrograph with high-power view of the photoreceptor layer shows normal stacking of disks with minor artifactual splaying. The splaying of disk segments and separation of rods is identical to that seen in control noninjected eyes. (Counterstained with lead citrate and uranyl acetate, X25,000). (D) Electron micrograph view of the outer nuclear layer (O) shows normal nuclear chromatin pattern and outer limiting membrane (arrows). (Counterstained with lead citrate and uranyl acetate, X4,000). (E) Electron micrograph view of the inner ganglion layer shows inner limiting membrane (arrows). (Counterstained with lead citrate and uranyl acetate, X7,500).
withdrawn. Next, the pelleted liposomes were disrupted by freezing and resuspending in 1 ml distilled water. Drug concentration was then determined by high-pressure liquid chromatography for ganciclovir and high-pressure liquid chromatography and/or ultraviolet absorption at 254 nm for 2'-nor-cyclic GMP.

Spectrophotometric analyses of 2'-nor-cyclic GMP within liposome fractions were performed by diluting the samples with chloroform-methanol (2:3 vol/vol) and measuring ultraviolet absorption at 254 nm. The concentration of 2'-nor-cyclic GMP was calculated from the A-254 value, the molar absorbance of the compound at 254 nm (13.7 x 10^3), and the molecular weight of the compound was 484.25. High-pressure liquid chromatographic analysis of either ganciclovir or 2'-nor-cyclic GMP was performed by diluting an aliquot of the chloroform-methanol diluted sample and performing chromatography on a carbon-18 column for ganciclovir or a Varian AX-10 analytical anion exchange column for 2'-nor-cyclic GMP using isocratic elution with 80 mM KPO_4 (pH 3.4) at a flow rate of 2 ml/min for each drug. The amount of drug was determined from the respective area under the peak that was read against a standard curve constructed by chromatographing known amounts of each compound under the same conditions. Ganciclovir encapsulation was carried out in the same fashion as 2'-nor-cyclic GMP with these modifications: 90 mM ganciclovir was dissolved in sterile water and titrated with HCL to a final pH of 1.2 before admixture with the lipid chloroform preparation. In the drying phase, the sucrose solution contained 40 mM of lysine and 210 mM of sucrose.

**RESULTS**

All experiments were repeated two to four times (Table 1). The vitreous remained clear throughout the postinjection period in all 25 rabbits. The 11 eyes that received 10 μg of drug had normal ERG findings (amplitude and latency) before and after the intraocular injection at 30- and 60-day time points (Fig. 1). Light microscopy and electron microscopy displayed normal retinal morphology at all time points (Figs. 2, 3, 4, 5). The retinas were observed to be attached and of normal appearance in 10- and 20-μg doses. At the 20-μg dose, the electroretinogram was normal at all time points. Mild changes consisting of vacuolization at the level of the retinal pigment epithelium were seen first at 1 week by light microscopy; the histology had been normal before this. At the 2-month time point, these changes resolved. The electroretinogram showed severe loss of amplitude after the first week after injection at both the 50- and 100-μg doses. The retina lost transparency at 30 days. The 17 eyes that received 50 and 100 μg showed toxic changes at 1 week, 1 month, and 2 months. Toxic changes observed at 50-μg doses varied from vacuolization of the retinal pigment epithelium and loss of height of the photoreceptor outer segments to severe loss of the entire outer retina. These were not seen 1 and 3 days after injection but became prominent at 1 week, and severe toxicity was seen at the 1- and 2-month time points. At higher doses (100 μg), the toxic effects became evident at the level of the retinal pigment epithelium earlier (3 days) with destruction of the outer half to outer 75% of the retina seen, beginning at the 1-week time point.

Based on our studies, the intraocular therapeutic index [maximum nontoxic dose (μg/cc)/dose (μg/cc) required to inhibit 50% HCMV colony formation] is 10^-.5 = 20.
Drug capture efficacy was 89% for 2'-nor-cyclic GMP within liposomes. The drug release half-life in vitro was 1,000 hours for 2'-nor-cyclic GMP. Ganciclovir drug capture efficacy was 42%, and it had a drug release half-life of 13 hours in vitro.

**DISCUSSION**

CMV infections are a major cause of morbidity and mortality in the immunocompromised host. They may present as various pathologic entities, but retinitis is the most common and requires indefinite treatment in the patient with AIDS. DHPG (ganciclovir) is commonly used in the treatment of CMV infections in the immunocompromised patient but has many side effects, particularly bone marrow toxicity. Another compound, phosphonoformate (foscarnet) has also been used in the therapy of HCMV retinitis with good success; however, the compound has disadvantages that include renal toxicity, anemia, electrolyte imbalance, tremor, and nausea. Recognizing that CMV retinitis may be diagnosed in the absence of systemic disease and recognizing the disadvantages of systemic toxicity and daily intravenous therapy with ganciclovir or foscarnet, alleviation of these concerns might result from use of local intravitreal preparations in the repository form which could be administered at infrequent intervals.

Although 2'-nor-cyclic GMP is a potent and selective inhibitor of CMV replication in vitro, the compound's mode of antiviral action is unknown but may involve inhibition of viral DNA polymerase. The ID50 concentrations of ganciclovir and 2'-nor-cyclic GMP to inhibit HCMV infection in tissue culture are 0.43 μmol/l and 2 μmol/l, respectively. Therefore, 2'-nor-
cyclic GMP and ganciclovir are comparably potent anti-CMV agents. Our results indicate that a 10-μg dose of 2’-nor-cyclic GMP, which is greater than 20 times the concentration required to inhibit CMV plaque formation by 50%, is not toxic to the rabbit retina by light microscopy, electron microscopy, and electrophysiologic testing for up to 8 weeks. Intravitreal injections of 2’-nor-cyclic GMP greater than 20 μg/0.1 ml resulted in severe toxic effects on the outer retina, especially the photoreceptor layer. The ERG results paralleled the morphologic findings at toxic and nontoxic doses at 1 month and 2 months. ERGs not performed before this, because nonspecific ERG changes were seen after intraocular injection of any substance.

We have shown that 2’-nor-cyclic GMP has a prolonged release time in a multivesicular liposome system that can be used to increase the intraocular time of residence of this compound. This will likely allow for a more favorable toxicologic profile. Studies by our group have indicated that the multivesicular liposome system used in this study is not toxic to the rabbit retina. We have found that polar derivatives that are water soluble and compounds of high molecular weight are well retained within a multivesicular liposome system. At neutral pH, ganciclovir is poorly water soluble, and the encapsulation efficiency in the aqueous phase of liposomes is low. The safe intravitreal level of ganciclovir in rabbit is between 50 and 200 μg per injection.23,24 Although ganciclovir is much more water soluble at pH 11,22 the lipids used in liposomes are hydrolyzed at this pH, and injection of highly alkaline substances into the eye may be associated with toxic problems. Further studies of polar drugs such as 2’-nor-cyclic GMP encapsulated within the aqueous phase of liposomes are warranted because such compounds, suitably encapsulated in liposome, may prove to be a useful therapeutic modality for the local intravitreal therapy of CMV and other forms of viral retinitis.

Key Words
antiviral drugs, CMV retinitis, AIDS, 2’-nor-cyclic GMP, retina

References
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